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(54) Title: GENERATION OF STABLE DIMERS WITH REVERSIBLE BIOTIN-BINDING ACTIVITY

(57) Abstract: Avidin is mutated by replacing the Trp110 with Lys, and streptavidin is mutated by replacing the Trp120 with Lys. Both of these mutant proteins produce a stable dimer. These stable dimers exhibit reversible biotin-binding properties. The mutant avidin and streptavidin can be used in all known avidin/streptavidin-biotin systems and purification processes, with the added advantage that the avidin/streptavidin and biotin may be eventually separated.

GENERATION OF STABLE DIMERS WITH REVERSIBLE BIOTIN-BINDING ACTIVITY

Field of the Invention

The present invention relates to avidin or streptavidin having a tryptophan to lysine mutation to generate stable dimers with reversible biotin-binding activity.

Background of the Invention

Avidin from chicken egg white (SEQ ID NO:1) and bacterial streptavidin (SEQ ID NO:2) share a high affinity for the vitamin biotin (K_a ~ 10^{15} M^{-1}), although their primary structures are not very well conserved (~35% homology). Despite the relatively low sequence homology, avidin and streptavidin share the same tertiary fold, similar tetrameric quaternary structures, and a nearly identical arrangement of amino acids within their respective biotin-binding pockets (Livnah et al, 1993a; Hendrickson et al, 1989; Weber et al, 1989; Pugliese et al, 1993).

Fibropellin is another protein which harbors a C-terminal domain similar in sequence to avidin and streptavidin. Fibropellins are epidermal growth factor (EGF) homologues found in the hyaline layer of the extracellular matrix in sea urchin embryos. Of particular interest is a fibropellin which consists of repeated EGF-like N-terminal domains and, unexpectedly, a single avidin-like C-terminal domain (Bisgrove et al, 1995). However, neither the function nor the three-dimensional structure of the avidin-like domain

is known, nor is it known whether the domain is capable of binding biotin. The amino acid residues of fibropellin are homologous to the biotin-binding residues of avidin and streptavidin and most of them appear to be conserved (Hunt et al, 1989). The most remarkable alteration is the replacement of Trp110 in avidin (equivalent to Trp120 in streptavidin) with lysine in fibropellin.

Proteins are comprised of amino acids connected to one another by amide bonds or peptide bonds. Native proteins are folded into precise and well-defined structures, which folding often depends on the particular sequence of amino acids. Substitution of amino acids, particularly in the active site of the protein, can affect the protein folding and the protein activity. To minimize changes in characteristics of the proteins, conservative amino acid substitutions are made. Conservative substitutions may be defined herein as exchanges within one of the following five groups:

I. Small, aliphatic, non-polar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly

II. Polar, negatively charged residues and their amides:

Asp, Asn, Glu, Gln

III. Polar, positively charged residues:

His, Arg, Lys

IV. Large, aliphatic non-polar residues:

Met, Leu, Ile, Val, Cys

V. Large aromatic residues:

Phe, Try, Trp

Within the foregoing groups, the following substitution are considered to be "highly conservative":

Asp/Glu

His/Arg/Lys

Phe/Tyr/Trp

Met/Leu/Ile/Val

Previous studies on the structure-function relationship in the avidin/streptavidin-biotin systems used a variety of different approaches in examining how flexible one can be in modifying different amino acids in the binding site while still conserving the property of biotin binding. These include chemical modification studies (Gitlin et al, 1987, 1988a, 1988b, 1989, 1990), X-ray analysis (Livnah et al, 1993a and 1993b), and sequence comparisons with naturally occurring, biotin-binding proteins, e.g., different types of streptavidins (Bayer et al, 1995) and avidin-related proteins (Keinänen et al., 1994). The results indicated that there is little compromise regarding the types and positions of binding-site residues. Most of the binding site residues are strictly conserved in order to maintain the property of strong binding to biotin. However, considerable flexibility can be applied in modifying residues outside of the binding site. In this regard, only about 35% of the primary structure is conserved between avidin and streptavidin. Interestingly, streptavidin has 25 alanines compared to only 5 in avidin,

perhaps indicating that the bacterial molecule evolved via a natural process of "alanine scanning", which may have led to improved properties.

As structural and potentially functional entities, both the avidin and streptavidin tetramer can conceivably be divided into three different types of dimer pairs (Livnah et al, 1993a). Functional dimers would presumably be formed between monomers 1 and 2 and between monomers 3 and 4, numbered according to Livnah et al (1993a), wherein the conserved Trp110 of one avidin monomer (Trp120 in streptavidin) is part of the biotin-binding pocket of its affiliate and *vice versa*. Trp110 is also noteworthy in another sense: this particular residue has a remarkable effect on the stability of the avidin tetramer (Livnah et al, 1993a).

Mutations of W120 in streptavidin have been described previously. In one study, Chilkoti et al (1995), the conservative mutation of this residue to phenylalanine resulted in a reduction of the binding constant for 2-iminobiotin by two orders of magnitude. Its replacement by alanine caused a more severe drop in the affinity of the mutated protein for biotin itself, to a measurable value (1.1×10^{-7} M), reminiscent of the affinity constant estimated for immobilized monomeric avidin ($K_d = 10^{-7}$ M) (Green et al, 1973; Kohanski et al, 1990). Both of these streptavidin W120 mutants were reported to assemble into tetramers.

Sano et al (1997) described the construction of a mutated form of streptavidin, in which the entire 8-residue loop connecting β-strands 7 and 8 was deleted. W120 was among the residues contained in the deleted loop. The resultant protein was shown to form a soluble dimer in the presence of biotin. Since the entire loop was deleted, it is not clear what consequences this would have on the flanking residues which normally form the two separated β-strands in question. Presumably, the residues immediately adjacent to the deleted loop would rearrange into a loop, hence detracting from the lengths of one or both strands. In any case, the present inventors observed that the stability of the deletion mutant in dimeric form was contingent upon the presence of biotin, the absence of which resulted in the gradual dissociation into inactive monomers.

Biotin binding of avidin and streptavidin has been useful in immunoasssays and purification studies. Avidin can also be used as a template for forming dimers and promoting protein-protein interactions required for signal transduction. However, conventional biotin binding of avidin or streptavidin is not readily reversible, which makes purification from recovery of biotinylated proteins somewhat cumbersome.

Summary of the Invention

It is an object of the present invention to provide modified avidin or streptavidin molecules having reversible biotin-binding activity.

It is another object of the present invention to mutate a tryptophan to a lysine in avidin or streptavidin.

Sea urchin fibropellin was used as a conceptual template for mutation of designated conserved tryptophan residues in the biotin-binding sites of egg white avidin and bacterial streptavidin. The homologous tryptophan-to-lysine (W → K) mutations of both avidin and streptavidin bound biotin and biotinylated material. Their affinity for biotin was significantly reduced, from K_d ~ 10^{-15} M of the wild-type tetramer down to K_d ~ 10^{-8} M for both W → K mutants. The binding of these mutants to immobilized biotin matrices could be reversed by the presence of free biotin.

Mutating either avidin or streptavidin by replacing the Trp110 with Lys in avidin (SEQ ID NO:3), or replacing Trp120 with Lys in streptavidin (SEQ ID NO:4), produced a stable dimer in both proteins. Both of these stable dimers exhibited reversible biotin-binding properties.

Brief Description of the Drawings

Figure 1 shows reversibility of biotin-binding activity of the W → K mutants of avidin and streptavidin. Both ELISA and IASyS protocols were used, and the results are expressed as % reversibility (mean ± standard error). Avidin and streptavidin were used as negative (irreversible) controls, and nitro-avidin was used as a positive control.

Figure 2 shows FPLC gel filtration profile of the

W → K mutants of avidin and streptavidin in the presence and absence of free biotin. A commercial Superose-12 column was used for separation. Human immunoglobulin (IgG), bovine serum albumin (BSA), avidin (Av), ovalbumin (Ov), and cytochrome c (Cyt c) were used as markers to calibrate the column. The inset shows the observed peak for Avm-W110K. Very similar, well-defined peaks were also obtained for Savm-W120K and for both mutants in the presence of biotin. The migration pattern of both mutants was consistent with a dimer, whether in the absence or presence of biotin.

Figure 3 shows a thermostability analysis of avidin, streptavidin and their W → K mutants in the presence of SDS. Samples in the presence or absence of free biotin were dissolved in SDS-containing sample buffer, incubated for twenty minutes at the indicated temperatures, and subjected to SDS-PAGE. The ratio of tetramer-to-monomer was determined by densitometry tracings, and the results were graphed as a function of temperature (Bayer et al, 1996b).

Figure 4 shows sensitivity of the W → K mutants of avidin and streptavidin to proteolytic digestion by proteinase K. The mutants of native proteins, in the presence or absence of biotin, were mixed with a 1:50 ratio (wt/wt) of proteinase K to target protein, and samples were taken at the designated time intervals. The samples were dissolved in SDS-containing sample buffer, boiled for 120 minutes, and subjected to SDS-PAGE. The values represent the relative percent of intact monomer observed in the sample, graphed as a function of time.

Detailed Description of the Invention

Three different proteins, egg white avidin, bacterial streptavidin, and sea urchin fibropellin, exhibit moderate levels of conservation in their primary amino acid sequences (Hunt et al, 1989). Avidin and streptavidin bind biotin with the highest known biological interaction between a protein and a ligand (Green, 1990), which makes them particularly useful for immunoassays and for protein purification. Their three dimensional structures have been determined, and the residues of the protein which interact with biotin have been characterized. The structure and function of the avidin-like domain of fibropellin are unknown, and a recombinant form has yet to be expressed. Despite the striking resemblance of its primary structure to both avidin and streptavidin, it is not yet known whether or not fibropellin binds to biotin.

Most of the important biotin-binding residues in avidin and streptavidin are conserved in fibropellin (*cf.* Table 1). Several discrepancies, however, can be noted. For example, a binding-site serine residue in both avidin and streptavidin, which forms a hydrogen bond with the carbonyl carbon of the biotin ureido ring, is substituted in fibropellin by an aspartic acid. Aspartic acid, however, is also capable of forming a hydrogen bond, and one could envision a conservative substitution in this case. Much more intriguing is the replacement of two tryptophans, which contribute to the aromatic cage of the binding pocket. One of

these tryptophans is replaced by an arginine and a second by a lysine.

Table 1
Conserved Binding-Site Residues in Avidin and Streptavidin and Their Analogues in Sea Urchin Fibropellin

Avidin	Streptavidin	Sea Urchin Fibropellin[a]
N12	N23	N947
S16	S27	D951[b]
Y33	Y43	Y967
T35	S45	T969
W70	W79	R1002
T77	T90	T1009
F79	W97	W1011
W97	W108	W1028
W110	W120	K1041
N118	D128	D1049

[a] Fibropellin residues were numbered according to GenBank accession number L08692. Analogous residues in the other three fibropellins (L07045, L33861 and L33862) are identical, except that the residue corresponding to tyrosine 33 in avidin is histidine in clones L33862 and L07045.

[b] Unconserved residues in fibropellin are underlined.

It is believed that the role of the avidin-like domain may be to form dimers and thereby promote protein-

protein interactions required for signal transduction. Avidin seems to serve as a template for such processes, since the dimer afforded by the W110K mutation is a stable one which exhibits a multiplicity of hydrogen bonds across the relevant 1 → 4 interface. Nevertheless, hydrogen bonds are relatively more easy to dissociate than other kinds of bonds, as demonstrated for the W110K mutant dimer, which reverts to the monomer in the presence of SDS. In any case, the exact nature of interaction among fibropellin components, including residual biotin-binding activity, depends on the microenvironment and structure of the native protein.

Using this information, the present inventors tried to replace either or both of these tryptophan residues in avidin to see whether the mutant proteins could be expressed in functional form. The double mutant appeared to be inactive and failed to bind to either iminobiotin or biotin-agarose. The W70R mutant appeared to exhibit relatively low levels of binding to both columns, but sufficient protein for biochemical analysis could not be prepared at that time.

Avidin-W110K was successfully cloned and expressed. Also, the cognate mutant from streptavidin, namely Savm-W120K, was prepared and examined.

Replacement of a tryptophan by a lysine is clearly an unorthodox substitution. It is surprising that such an extraordinary mutation did not seem to interfere significantly with the expression and purification of the mutant protein,

which probably reflects the remarkable stability of the native protein.

The W → K mutation had two major effects on the avidin and streptavidin molecules. In both proteins, stable dimers were generated, and the affinity constant for biotin was markedly reduced, although it was still stronger than most intermolecular interactions. More importantly, the interaction of Avm-W110K and Savm-W120K with biotin was reversible.

Conservation of the activity of the mutant proteins was surprising because of the non-conservative nature of the substitution. This is particularly true in light of the different results for more conservative substitutions of the same tryptophan by phenylalanine and alanine, as reported by Chilkoti et al (1995). However, formation of the dimer was not unreasonable. In both native proteins, the respective tryptophan contributes to the binding site of one monomer by an extended pair of β strands from its symmetry-related neighbor, thus comprising a major component of the "functional" 1 → 2 interface of the tetramer. Nevertheless, in order to form a dimer from a tetramer, two different interfaces must be impaired, the inference being that both the 1 → 2 and the relatively weak 1 → 3 interfaces would be affected by the single, dramatic mutation of tryptophan (100 in avidin, 120 in streptavidin) to lysine. This would result in the formation of stable "structural" dimers, in which the extensive network of hydrogen-bonding interactions along the

1 → 3 interface would presumably be retained. However, this network of hydrogen bonds was sensitive to SDS, and its presence caused dissociation of the dimer into the monomer even at room temperature.

Modifying of both homologous tryptophans (W110K and W70R) in avidin led to an inactive binding protein.

The tryptophan-to-lysine mutations of the present invention bind biotin reversibly and are stable to storage for long periods of time. Thus, these mutant proteins are useful in purification studies in which recovery of biotinylated molecules, such as biotinylated proteins, is desired. The mutant proteins are added to a solution or suspension of biotinylated molecules, where the mutant avidin or streptavidin proteins bind to the biotinylated molecules. This aggregate is removed from the solution or suspension, and biotin is added to release the mutant protein from the biotin-mutant bond. Thus, the desired molecule can be obtained.

Mutant avidin and streptavidin can be used in other types of protein purification. For example, the mutant avidin or streptavidin may be immobilized onto a chromatographic column. A target molecule is separately reacted with biotin to form a biotinylated target molecule. The biotinylated target molecule is then added to the mutant avidin/streptavidin-immobilized chromatographic column. Due to binding of the avidin to the biotin, the biotinylated target molecule is then immobilized on this column. The target molecule can then be used to purify a second molecule

which is capable of binding to or interacting with the target molecule. The target molecule may, for example, be an immunoglobulin with the test molecule being a molecule to which the immunoglobulin is specific, or *vice versa*. Similarly, the target molecule may be a portion of a receptor, and the second molecule a ligand to which the receptor is specific, or *vice versa*. The target molecule may be other than a protein, such as an organic or inorganic molecule which is capable of binding to or reacting with specific second molecules.

When a solution or suspension of second molecules is added to the column, only those molecules recognized by the target molecule will bind to the immobilized target molecule. Thereafter, the second molecule may be released from the target molecule by appropriate altering of the conditions, as is well known in the art of affinity chromatography. Because the mutant avidin/streptavidin binds to the biotin more strongly than the target molecule binds to the second molecule, the second molecule may be released from the first molecule without causing the target molecule to also be released from the column. Subsequently, the biotinylated target molecule can be released from the column in order to regenerate a mutant avidin/streptavidin column, which can be used again in the future with other biotinylated molecules. The biotinylated target molecule may be released by adding biotin to the column, or by otherwise altering the conditions, such as pH conditions, in such a way as to allow the

reversible bond between the mutant avidin/streptavidin dimer and the biotin of the biotinylated target protein. The general method of purifying proteins using an avidin biotin system is disclosed, for example, in Katz U.S. Patent No. 4,253,995. The novelty of the present method is in the novel avidin/streptavidin mutant which is used and the capability of thereafter removing the biotinylated target molecule from the column in order to regenerate an avidin column for future use and to regenerate the biotinylated target protein for future use.

Alternatively, in the above process, it may be desirable to maintain the target molecule second molecule conjugate. In this case, the entire conjugate of biotinylated target molecule-second molecule can be removed from the column due to the reversible association of the mutant avidin/streptavidin on the column with the biotin of the biotinylated target molecule. This release may be accomplished by the addition of additional biotin or by otherwise altering conditions to allow the release of the biotinylated target molecule from the mutant avidin/streptavidin column. This will provide a purified biotinylated target molecule-second molecule conjugate or reaction product.

Such a column of mutant avidin/streptavidin can also be used to isolate biotinylated molecules from a mixture of such biotinylated molecules with other non-biotinylated molecules. In this embodiment, the mixture is added to the

mutant avidin/streptavidin column, and only the biotinylated molecules will remain fixed to the column. The biotinylated molecules may then be removed from the column due to the reversible nature of the binding allowed by the peculiar nature of the mutant avidin/streptavidin which is used.

The mutant avidin and streptavidin can also be used in assays based upon biotin-avidin or biotin-streptavidin binding. In this case, using transferase as an example, mutant avidin or streptavidin resin is added to a transferase assay reaction mixture in which the peptide substrate has been tagged with biotin. The biotinylated substrate will bind to the mutant avidin/streptavidin of the resin. This binding assists in increasing the product's apparent molecular weight for an ultrafiltration separation. Ultrafiltration is used to separate tagged product from unreacted tagged substrate. The tagged product then can be recovered free of mutant avidin or mutant streptavidin by adding excess biotin (or other appropriate conditions) to release the tagged product. An ultrafiltration assay procedure is disclosed in detail in Huang, U.S. Patent No. 5,869,275, the entire contents of which are hereby incorporated by reference.

Another assay for proteins involves determining the amount of an adherent protein in a sample by contacting a sample with a support medium having high affinity for the adherent protein. A description of such an assay is found in Sipe et al, U.S. Patent No. 5,536,640, the entire contents of which are hereby incorporated by reference.

It should be understood that the present invention is not limited to the mutation of avidin from chicken egg-white or bacterial streptavidin from a specific strain of *Streptomyces avidinii*, i.e., the proteins with the specific sequence of SEQ ID NO:1 and SEQ ID NO:2. It is known, for example, that avidin is also found in the egg-white of other birds, and possibly also in the egg white of reptiles. Similarly, streptavidin may be found in other strains and species of bacteria. The proteins found in these other sources may have slightly different amino acid sequences, although the sequences in the biotin-binding regions are generally substantially the same. Thus, it would be fully expected that if the tryptophan in the position corresponding to Trp110 of SEQ ID NO:1 or Trp120 of SEQ ID NO:2 is changed to a lysine, the resultant molecule will also form a dimer, rather than a tetramer, which will allow reversible association with biotin. Furthermore, analogs of avidin and streptavidin are known in the art which retain their binding to biotin. Such analogs can also be mutated in accordance with the present invention in order to achieve the desirable properties described herein.

The sequences for chicken egg white avidin and *Streptomyces avidinii* streptavidin were taken from GenBank Accession L27818 and GenBank Accession X03591, respectively. The first 24 amino acids of each, which are the signal sequence or leader peptide, have been omitted. It should be understood, however, that in streptavidin the next 13 residues

(residues 1-13 of SEQ ID NO:2) are often cleaved by a protease, as are the final 23 residues (residues 136-159 of SEQ ID NO:2). Thus, while the original full sequence of streptavidin is 159 residues after cleavage of the signal sequence, the final size of the proteolytically truncated streptavidin, called "core" avidin, is 123 amino acid residues. The N- and C-termini which are cleaved to form core streptavidin are irrelevant to the structure and biotin binding. The final, trimmed core streptavidin, is analogous to avidin (128 residues) in sequence, structure and biotin-binding properties.

Accordingly, the terms "avidin" and "streptavidin" as used in the present claims is intended to include not only the chicken egg-white avidin and bacterial streptavidin of SEQ ID NOs:1 and 2, but also avidin and streptavidin from other species and other analogs of avidin or streptavidin which bind to biotin in a manner similar to that of the avidin and streptavidin of SEQ ID NOs:1 and 2. The terms "avidin" and "streptavidin" are also intended to include fragments of avidin and streptavidin which maintain the biotin-binding properties of the full-length protein, such as, for example, "core" streptavidin. Reference to tryptophan 110 with respect to avidin and tryptophan 120 with respect to streptavidin is intended to refer to the tryptophan at the position specified by the numbering of SEQ ID NOs: 1 and 2, as well as the corresponding position of whatever avidin or streptavidin is used.

It should further be understood that the mutant avidin or streptavidin of the present invention may be used in any avidin/streptavidin-biotin system or purification process in which native avidin or streptavidin can be used. Each such system or process will be improved by the fact that the avidin/streptavidin-biotin bond is reversible when substituting the mutants of the present invention.

EXAMPLE:

Materials and Methods

Materials

Biotin-agarose and 2-iminobiotin-agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Egg-white avidin was a gift of Belovo Chemicals (Bastogne, Belgium) or STC, Inc. (Winnipeg, Canada). Nitro-avidin was prepared as previously described (Morag et al, 1996). Streptavidin was provided by S.p.a., Inc. (Milan, Italy). Rabbit avidin and streptavidin antibodies were acquired from DAKO A/S (Glosstrup, Denmark). Goat anti-rabbit IgG alkaline phosphate conjugate and the low-range SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA). JM109 and DH10Bac *E. coli* strains were used in cloning procedures. Mutants were produced in baculovirus infected Sf9 insect cells.

Recombinant Bacmids and Baculoviruses

Multiple sequence alignment (not shown) of avidin, streptavidin and sea urchin fibropellins was carried out using CUSTAL X (Thompson et al, 1997), and the GCG package (Genetic Computer Group, Madison, WI), using default parameters, was employed to calculate the theoretical molecular weights for the mutant proteins.

Avidin and streptavidin cDNAs were mutated by the megaprimer method (Sarkar et al, 1990), using pGEMAV as an avidin template (Airenne et al, 1994) and the streptavidin gene from *S. avidinii*, prepared as previously described (Bayer et al, 1995). After a second PCR amplification, the product for the avidin mutant was digested with BglII and HindIII, and the product for the streptavidin mutant was digested with BamHI and HindIII. Both digests were then extracted from agarose and cloned into BamHI/HindIII-treated pFASTBAC1 to construct the recombinant vectors, pFδ9 (avidin mutant Avm-W110K), pFδ10 (Avm-W70R), pFδ17 (Avm-W70R&W110K) and pFSAK (streptavidin mutant Savm-W120K). The resultant vectors were transformed into JM109 cells to construct the recombinant baculoviruses. The correct nucleotide sequence was confirmed in each case by dideoxynucleotide sequencing with an automated DNA sequencer. The preparations of the recombinant viruses were finally completed according to the manufacturer's instructions for the Bac-To-Bac™ Baculovirus Expression System (GIBCO BRL, Life Technologies, Gaithersburg, MD).

The primary virus stocks were amplified for large-scale production of the mutants (Avm-W110K, Avm-W70R, Avm-W70R&W110K, and Savm-W120K), and the titres of virus stocks were determined by a plaque assay procedure (O'Reilly et al, 1994).

Preparation of Mutant Proteins

Production of mutant proteins was carried out essentially as previously described for recombinant avidin (Airenne et al, 1997), with the exception that cell density in different experiments varied from 1 X 10^6/ml to 2 X 10^6/ml and the m.o.i. from 0.1 to 5. In experiments where mutant proteins were purified, infections were carried out with cells that had been transferred to biotin-free medium (Gibco BRL, Cat. #041-94100).

Purification of the mutant proteins from the corresponding cell extract was performed on 2-iminobiotin-agarose as reported by Airenne et al (1997). Following cell lysis, the soluble fraction was brought to pH 11 and applied to a 2-iminobiotin-agarose column. The mutants were eluted using 50 mM ammonium acetate (pH 4/0.1 M NaCl). Later in the study, purification of Avm-W110K on a biotin-agarose column proved superior to that using 2-iminobiotin. In this case, the pH of the cell extract was not altered prior to application on the column. Adsorbed material was eluted either using 0.2 mg/ml biotin in 50 mM ammonium acetate (pH 4/0.1 M NaCl) or by 0.4 M acetic acid.

Biotin-Binding Assays

Biotin binding was initially assessed by dot-blot assay, using a modification of procedures described in Bayer et al (1988 and 1996a). Briefly, successive dilutions of biotinylated BSA were applied to dot blots. The blots were quenched using BSA, and an avidin, streptavidin or mutant test sample (1 μl of a 20-μg/ml solution in PBS) was applied. The interaction was carried out at 23°C for 30 minutes. The blots were washed with PBS-0.5% (v/v) Tween solution and stained immunochemically as described below.

A complementary method was used to achieve defined affinity constants for the mutants. An IASyS optical biosensor (Affinity Sensors, Cambridge, UK) and commercially available biotin-aminosilane cuvette (FCB5401) were used to obtain the kinetic measurements of the interaction between Avm-W110K or Savm-W120K and biotin. Different concentrations of mutant protein were allowed to bind to the biotinylated surface, and the affinity constants were calculated using the FASTfit software, developed by Affinity Sensors. Experiments were performed in PBS, containing 1 M NaCl, and cuvette regeneration was accomplished by 20 mM HCl.

Reversibility Assay – Competitive Biotin-Binding ELISA Assay

Microtiter plates were coated with 1 μg of biotinylated BSA per well in 100 μl coating buffer (15 mM sodium carbonate buffer, pH 9.6). The plates were incubated overnight at 4°C (or, alternatively, at 37°C for 2 hours), washed with PBS, quenched using 0.5% BSA in PBS at 37°C for 1

hour, and washed again in Wash Buffer A (1 M NaCl and 0.5% v/v Tween 20 in PBS). Three concentrations (10, 20 and 50 ng/well in Assay Buffer A, consisting of 0.5% BSA, 0.5% Tween 20 and 1 M NaCl in PBS) of mutant were applied in sextuplicate to the wells. Native avidin was used as a negative control (i.e., no reversibility) and nitro-avidin (Morag et al, 1996) as a positive control (normally ~ 70% reversibility). The wells were washed with Wash Buffer A.

At this point, the microtiter plate contained different types of avidin or streptavidin or mutant bound through the biotin moiety of the adsorbed biotinylated BSA. The remainder of the assay was designed to test how much could be released using biotin.

Half of the appropriate wells (i.e., three wells each of the test samples) were incubated with 0.5 mM biotin dissolved in Assay Buffer A at 37°C for 1 hour, in an attempt to displace, if possible, the adsorbed protein from the biotinylated BSA. The plates were then washed three times with Wash Buffer A and once with PBS alone.

The amount of avidin, streptavidin, nitro-avidin or mutant remaining on the plates was determined immunochemically as described below. The percent reversibility of the interaction between the desired biotin-binding protein and the biotin ligand was determined according to the following equation:

$$\text{Reversibility (\%)} = 100(A - B)/A$$

where A = the amount of protein bound to the wells in the initial interaction, and B = the amount of protein remaining after subsequent interaction with free biotin.

A complementary reversibility assay was also devised, which employed the optical biosensor (IASyS) and commercial biotin cuvettes as described above. In this case, avidin, streptavidin or their mutants were allowed to bind to the biotin-aminosilane cuvette in PBS, containing 1 M NaCl. After careful washings, the binding was measured, and the binding buffer was saturated with biotin. The proteins were allowed to elute until the system reached an equilibrium level. After this, another extended wash was performed using the binding buffer. The residual amounts of bound protein, following the wash, were compared to those measured after the initial binding phase, and reversibility was determined according to the above equation.

Immunoassay

Avidin- or streptavidin-containing dot blots or plates were incubated with rabbit anti-avidin or anti-streptavidin immunoglobulin at 37°C for 1 hour. The antibody was diluted (1:5000 from a 10 mg/ml solution) in Assay Buffer B (0.5% BSA, 0.05% v/v Tween 20, 0.9% NaCl, and 0.05% w/v sodium azide in 50 mM Tris-HCl buffer, pH 7.75) to prepare final concentrations of 3 μg/ml for nitrocellulose strips and 330 ng/well for microtitre plates. The plates were again washed three times with Wash Buffer B (0.05% v/v Tween 20 in 50 mM Tris-HCl buffer, pH 7.75), and then incubated with anti-

rabbit-IgG-conjugated alkaline phosphatase (Jackson ImmunoResearch) at 37°C for 1 hour, diluted 1:5000 in Assay Buffer B. Following another three washes in Wash Buffer B and a single wash in PBS, Substrate Solution (10 mg *p*-nitrophenyl phosphate in 10 ml 0.1 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$) was added. Color development (405 nm) was measured at 15-minute intervals, using an EL309 Microplate Autoreader (Bio-Tek Instruments, Burlington, VT).

Sensitivity to Proteases

A sample (50 µl of a 1-mg/ml aqueous solution of avidin, streptavidin or mutant in the presence or absence of excess free biotin) was added to an equal volume of 100 mM Tris-HCl buffer, pH 7.8. To this solution, proteinase K (5 µl of a 0.2 mg/ml aqueous solution) was added. The reaction solution was incubated at 37°C. At designated time intervals, 20 µl samples were taken and stored at -20°C. To each sample, 10 µl of sample buffer were added, the samples were boiled and subjected to SDS-PAGE on 18% gels. The amount of intact avidin in the expected band was determined in each sample by densitometry and compared to that of an untreated control sample (defined as 100%). The results were graphed as stability to protease treatment (sample/control x 100%) versus time of reaction.

Miscellaneous Methods

SDS-PAGE and immunoblot analyses were as previously reported by Airenne et al (1997). For thermostability analysis, the protein samples were combined with sample buffer

and incubated at selected temperatures for 20 minutes before being subjected to SDS-PAGE (Bayer et al, 1996a) on 15% gels. The gels were stained using Coomassie brilliant blue.

Circular dichroism (CD) spectroscopy studies were carried out using a Jasco 715 circular dichrometer at the far-UV region. Measurements were performed in circular 1 mm cuvettes at 22°C in 100 mM potassium phosphate buffer, pH 7.4. Variance, caused by different concentrations, was corrected in final spectra.

The quaternary status of avidin, streptavidin or mutants were determined by fast-protein liquid chromatography (FPLC), performed on a Superose 12 column (Pharmacia) using an LKB HPLC system. Samples (40 μg in 100 μl of phosphate buffer in 0.65 M NaCl, pH 7.2) were applied, and chromatography was carried out at a flow rate of 0.5 ml/min, using the same buffer and ionic strength in the equilibration and running phases. The column was calibrated using bovine γ-globulin, bovine serum albumin, an egg-white avidin standard, ovalbumin, carbonic anhydrase, ribonuclease, and cytochrome c as molecular-weight markers.

Results

Synthesis and Purification of Mutant Proteins

Sf9 cells, infected with recombinant viruses, produced three separate protein bands in SDS-PAGE, which were detected by immunoblot analysis using avidin-specific antibody. The bands represent carbohydrate-free and two differently glycosylated avidin forms (Arienne et al, 1997).

These bands were not observed from cells infected by wild-type virus (not shown). Cell lysates were clarified by centrifugation (1000 g, room temperature, 5 minutes) and adjusted to pH 11/1 M NaCl, before being subjected to purification on 2-iminobiotin-agarose. The efficiency of such columns was less than 25% for Avm-W110K. The residual fraction was, therefore, subjected to purification using a biotin-agarose column, and the final combined yield surpassed 75%. The same high yield could also be achieved by purifying mutant samples with biotin-agarose alone. Efficient elution of the 2-iminobiotin purified Avm-W110K was achieved using pH 4 buffer. A biotin-containing solution was effective for elution of the mutant protein from biotin-agarose; elution using 0.4 M acetic acid was also possible but less efficient.

Savm-W120K was prepared by a similar procedure. Following SDS-PAGE and immunoblot analysis, only a single band, consistent with monomeric core streptavidin, was detected by streptavidin-specific antibody. The mutant protein was isolated on a 2-iminobiotin column and efficient elution of Savm-W120K was achieved using pH 4 buffer.

In contrast to successful purification of Avm-W110K and Savm-W120K the present inventors were unable to purify two other avidin mutants, Avm-W70R and Avm-W70R&W110K, using 2-iminobiotin or biotin columns.

Biotin-Binding Properties

Dot-blot assays revealed that both the purified Avm-W110K and Savm-W120K mutants bound biotin (not shown). In order to provide more quantitative data, the W → K mutants were subjected to interaction analysis using an IASyS apparatus and a biotin aminosilane cuvette. Using this system, a measurable dissociation constant (K_d value) for the interaction between Avm-W110K and biotin was determined directly from the binding curves to be 2.8×10^{-8} M (Table 2). A very similar value (2.7×10^{-8} M) was also obtained from the equilibrium response data. The equivalent values for Savm-W120K were 5.8 and 5.9×10^{-8} M. These results are shown in Table 2. Unlike biotin cuvettes adsorbed with native avidin, the mutant-adsorbed cuvettes could be regenerated completely by a 1-minute wash with 20 mM HCl.

Table 2

Affinity Constants between Immobilized Biotin and the Mutants, Determined Using an IASyS Optical Biosensor

Mutant	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_d (M) [a]	K_d (M) [b]
Avm-W110K	1,080,973	0.02918	2.7×10^{-8}	2.8×10^{-8}
Savm-W120K	117,992	0.0071	6.0×10^{-8}	5.8×10^{-8}

[a] The dissociation constant (K_d) was calculated from the k_{ass} (association rate constant), derived from association analysis using the plot of K_{on} against protein concentration, and the k_{diss} (dissociation rate constant), was calculated directly from dissociation curves.

[b] The dissociation constant was determined directly from the binding curves.

As shown in Fig. 1, the biotin-binding property of both W→K mutants was reversible. In fact, the extent of release of the mutant from the biotinylated surface was superior to that exhibited by nitro-avidin (22). The results for reversibility of binding were similar for both of the assay techniques used in this study.

Structural and Stability Properties of Avm-W110K and Savm-W120K

The secondary structure of Avm-W110K was studied by CD-spectroscopy. The far-UV spectra (data not shown) resembled that of the wild-type avidin (Green et al, 1966), suggesting that the β-barrel fold of the avidin mutant had remained intact.

In order to determine the quaternary status of the

l-filtration FPLC was performed (Fig. 2). In
:ant migrated as a single symmetrical peak
When compared to standards and a commercial
n, the molecular weight of the Avm-W110K
ined to be 31,200 daltons (average of three
iments). The calculated theoretical
of the monomer (without sugar) is 14,285
:arbohydrate moiety comprises about 10% of
reen, 1975; Hiller et al, 1987). According
:he best fit for the quaternary state of Avm-
the hypothetical molecular weight of which
ately 31,500 daltons. The corresponding
nt, Savm-W120K, gave a similar value of
nich is also consistent with a dimer. The
n failed to affect the apparent quaternary
ner mutant. Both Avm-W110K and Savm-W120K
whether in the presence or in the absence of
each case, mutation of Trp-110 in avidin or
idue (Trp-120) in streptavidin caused a
tetramer to a dimeric form of the mutant

E has previously been used to analyze the
ies of avidin, streptavidin (Airenne et al,
, 1996b) and a progressive series of avidin
arttila et al, 1998). Using this procedure,
d Savm-W120K were found to dissociate into
resence of SDS already at room temperature

(Fig. 3). Unlike the native proteins and reduced-charge mutants, the presence of biotin failed to stabilize the W → K mutants, and tetramers were not observed. These results are consistent with the FPLC data, which demonstrated the dimeric nature of both mutant proteins. In both cases, the respective dimer is less stable than the native tetramer, and the detergent appears to convert the dimer to the monomer.

The stability properties of both mutants were also studied by their susceptibility to proteinase K treatment. Compared to native avidin, Avm-W110K was extremely sensitive to protease treatment (Fig. 4). Within minutes, all of the avidin dimer was cleaved, and the resultant lower-molecular-weight bands were consistent with the previously described proteinase K-sensitive cleavage site in one of the loops (Ellison et al, 1995). Unlike native avidin, the presence of biotin failed to protect the mutant from rapid proteolytic cleavage. In contrast, the streptavidin mutant Savm-W120K, like the native protein, was completely stable to the action of proteinase K, both in the presence and in the absence of biotin.

Both W → K mutants of avidin and streptavidin were generally quite stable under normal conditions. One indication of such stability of the Avm-W110K mutant is the observation that a sample, stored at room temperature in the absence of preservatives for one month, was unaltered in its biotin-binding properties, as measured by optical biosensor analysis (IASyS) as described above (not shown).

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing form the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent application, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not any way an admission that any aspect, description or

embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various application such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning an range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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WHAT IS CLAIMED IS:

1. A mutant avidin or streptavidin wherein tryptophan 110 of avidin is substituted by lysine or wherein tryptophan 120 of streptavidin is substituted by lysine.

2. A mutant avidin or streptavidin in accordance with claim 1, consisting of a mutant avidin wherein tryptophan 110 is substituted by lysine.

3. A mutant avidin or streptavidin in accordance with claim 1, consisting of a mutant streptavidin wherein tryptophan 120 is substituted by lysine.

4. A reagent for reversible binding of biotin comprising the mutant avidin or streptavidin of claim 1 bound to a suitable insoluble carrier.

5. A purification method comprising:

contacting a biotinylated molecule with an insoluble reagent in accordance with claim 4, whereby the biotin of the biotinylated molecule will become bound by the mutant avidin or streptavidin of the insoluble reagent;

releasing and recovering the biotinylated molecule from the insoluble reagent.

6. A method in accordance with claim 5, wherein said releasing step is achieved by the addition of biotin.

7. A method in accordance with claim 5, wherein said biotinylated molecule is contacted with said reagent in admixture with other non-biotinylated molecules.

8. A method in accordance with claim 5, further including, between said contacting and said releasing steps, the steps of:

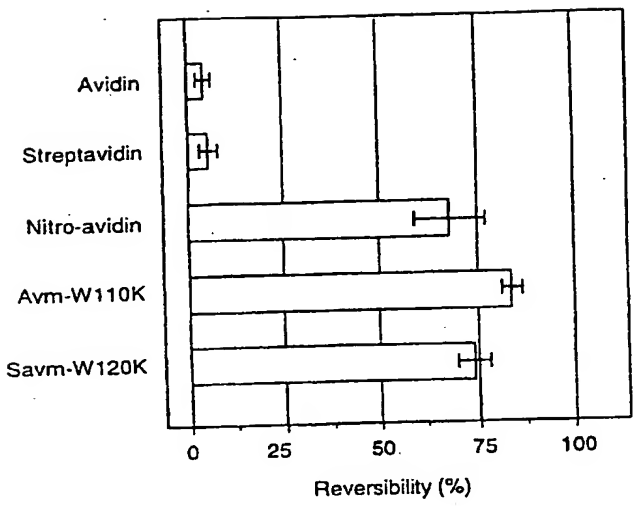
contacting said insoluble reagent-biotinylated molecule conjugate with a mixture including a second molecule which is capable of binding to, reacting with, or otherwise conjugating to said biotinylated molecule; and

releasing and recovering said second molecule from said biotinylated molecule.

9. A method in accordance with claim 5, further including, between said contacting and said releasing steps, the step of:

contacting said insoluble reagent-biotinylated molecule conjugate with a mixture including a second molecule which is capable of binding to, reacting with, or otherwise conjugating to said biotinylated molecule;

wherein said releasing and recovering step achieves the release and recovery of said biotinylated molecule-second molecule conjugate.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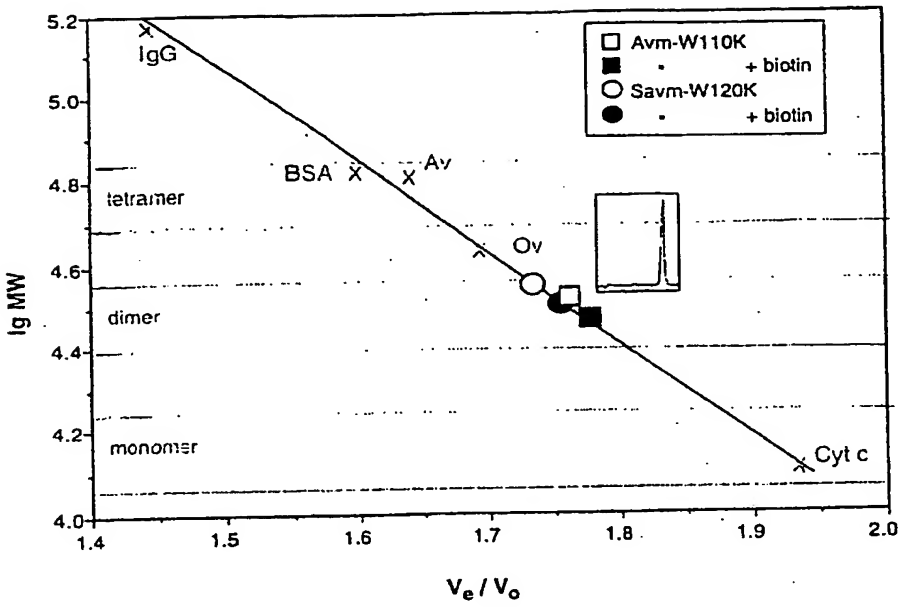
25
50
75
100
Reversibility (%)

Figure 1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tetramer
dimer
monomer
Avm-W110K
+ biotin
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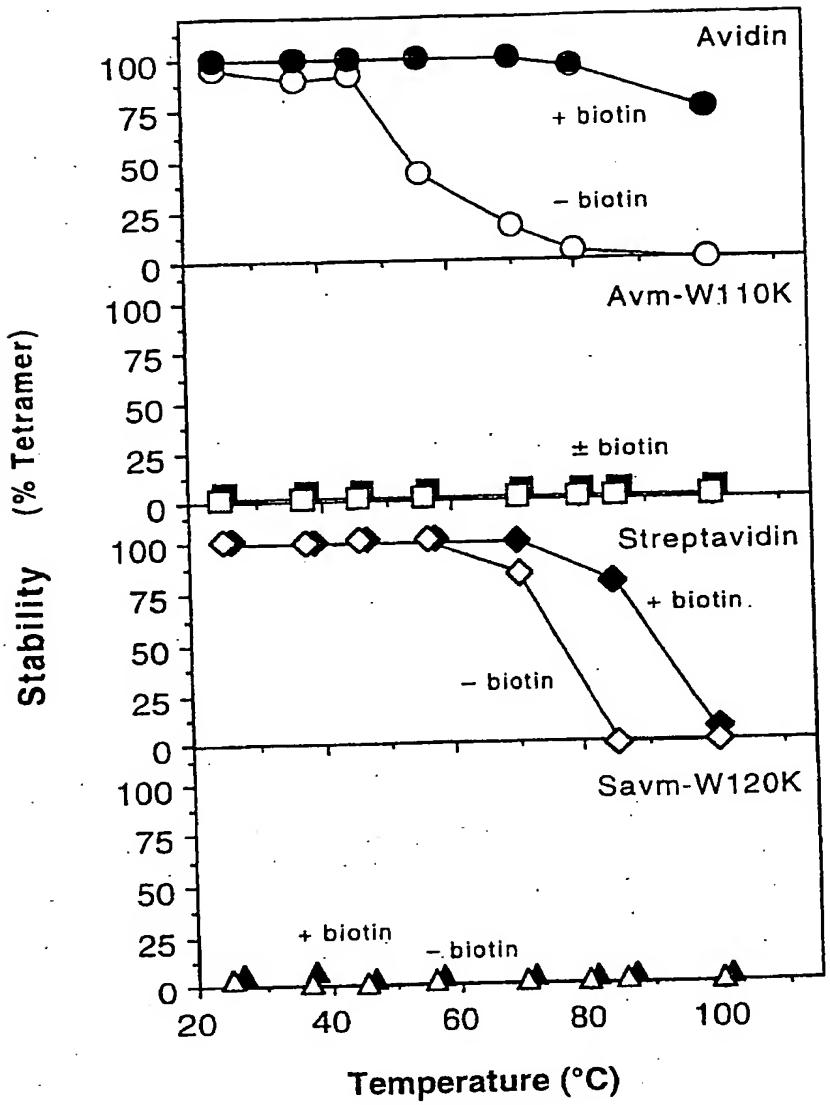
Ve / Vo

Figure 2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± biotin
Streptavidin
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– biotin
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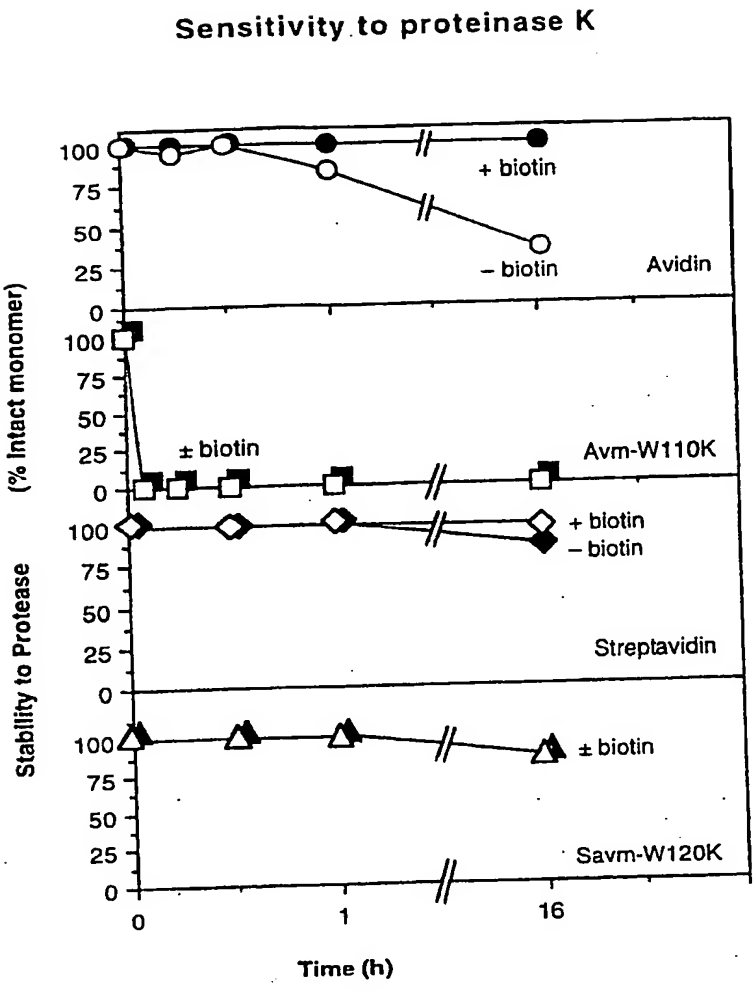
Temperature (°C)

Figure 3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+ biotin
– biotin
Avidin
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– biotin
Streptavidin
± biotin
Savm-W120K
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1
16
Time (h)

Figure 4

SEQUENCE LISTING

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BAYER, Edward A.
WILCHEK, Meir
LAITINEN, Olli H.

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Gly Gln Cys Phe Ile Asp Arg Asn Gly Lys Glu Val Leu Lys Thr Met
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50 55 60

Ala Thr Asp Gly Ser Gly Thr Ala Leu Gly Trp Thr Val Ala Trp Lys
65 70 75 80

Asn Asn Tyr Arg Asn Ala His Ser Ala Thr Thr Trp Ser Gly Gln Tyr
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Val Gly Gly Ala Glu Ala Arg Ile Asn Thr Gln Trp Leu Leu Thr Ser
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Gly Thr Thr Glu Ala Asn Ala Lys Lys Ser Thr Leu Val Gly His Asp
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Thr Phe Thr Lys Val Lys Pro Ser Ala Ala Ser Ile Asp Ala Ala Lys
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/18959

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/36 C07K14/465 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 24606 A (UNIV WASHINGTON) 15 August 1996 (1996-08-15) examples 1-3 ---	1-9
Y	WO 97 11183 A (UNIV BOSTON) 27 March 1997 (1997-03-27) examples 1-12 ---	1-9
P,X	LAITINEN OLLI H ET AL: "Mutation of a critical tryptophan to lysine in avidin or streptavidin may explain why sea urchin fibropellin adopts an avidin-like domain." FEBS LETTERS, vol. 461, no. 1-2, 12 November 1999 (1999-11-12), pages 52-58, XP000961177 ISSN: 0014-5793 the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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1 December 2000	08/12/2000

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PUGLIESE LUISA ET AL: "Three-dimensional structure of the tetragonal crystal form of egg-white avidin in its functional complex with biotin at 2.7 A resolution." JOURNAL OF MOLECULAR BIOLOGY, vol. 231, no. 3, 1993, pages 698-710, XP000972030 ISSN: 0022-2836 cited in the application	

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information on patent family members

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